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Issues in Biochemical Applications to Risk Assessment: Are Short-Term Tests Predictive of *In Vivo* Tumorigenicity?

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Introduction

My commentary is based on a study that we recently completed under the aegis of the National Toxicology Program (NTP). This study was an objective effort to compare results of *in vitro* tests, which measure mutagenicity in two different systems or clastogenic effects, either as chromosomal aberrations or as sister chromatid exchange, with chemicals that have been adequately tested for carcinogenicity (*1*). We were able to evaluate chemicals that have, in many cases, been the same batches that were used in the two rodent species for tumorigenicity studies. We were able to objectify the study by conducting the *in vitro* tests with protocols that were standardized to give reproducible results within and between laboratories. Most importantly, the assays were conducted on chemicals identified by code.

The value of the NTP tumorigenicity data base is derived from the fact that it is one of the few sources of results where animals are exposed under conditions that will allow for the identification of no evidence of carcinogenicity. There was a total of 73 chemicals in the study group, 44 of which were identified as carcinogens, in that they induced some pattern of tumorigenicity in at least one sex of either the rat or mouse strains that were studied. These tumorigenicity results were then compared both in a binary fashion, i.e., plus-minus basis, and in more depth, with the results from *in vitro* genetic toxicity tests.

The overall results of this study are given in Figure 1 to illustrate a couple of the important points. The results are divided into those that are mutagens or those that are nonmutagens; those that are carcinogenic mutagens and noncarcinogenic mutagens. The largest relative proportions of these 73 chemicals fall into the 3 groups represented by the mutagenic carcinogens and the nonmutagenic noncarcinogens and nonmutagenic carcinogens. The chemicals that induced mutagenicity in this figure are based solely upon the results of tests conducted in Salmonella. The substances that were

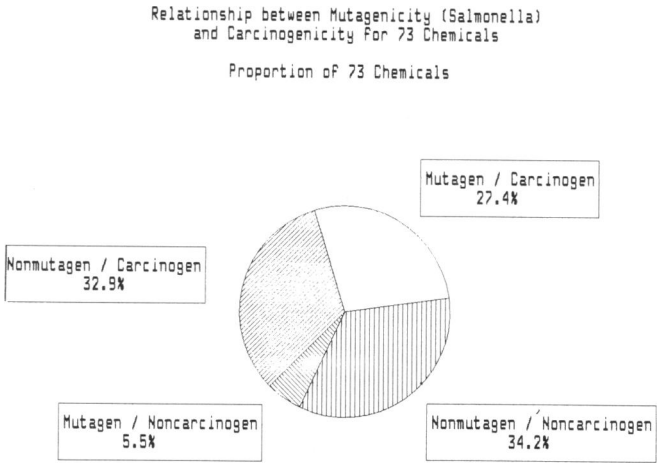

FIGURE. 1. Seventy-three chemicals that have been assayed for carcinogenicity in two rodent species were evaluated for ability to induce mutations in Salmonella. The proportions of the four conbinations of responses are presented.

mutagenic but not carcinogenic represented only approximately 20% of the total mutagens. Therefore, an important conclusion to be derived from this study is that the proportion of substances that are carcinogenic but nonmutagenic is far greater than has been reported from studies conducted elsewhere.

We believe that further evaluation of chemicals that represent mutagenic noncarcinogens can be constructively approached by looking at the potential genetic toxicity of these chemicals in *in vivo* systems. These same 73 chemicals currently are being evaluated for their clastogenic effects *in vivo*, also under code. We will test the possibility that *in vivo* genetic toxicity assays will be able to help to prospectively distinguish between substances that are in essence hazardous mutagens and those that may be nonhazardous mutagens because of the ability of the rodents to either metabolize or dispose of them in a manner that does not give them effective access to DNA.

The major problem then for the prospective use of *in*

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vitro tests is the inability to distinguish between the carcinogenic nonmutagens and the noncarcinogenic nonmutagens. This represents a major blind spot.

It has been proposed that some combination (battery) of *in vitro* assays would complement the Salmonella assay in the identification of carcinogens. Therefore, we did a fairly extensive comparison with another mutagenicity assay in mammalian cells, and assays for clastogenicity and sister chromatid exchange (SCE) induction. We also looked for the best of all the possible combinations in comparison to the Salmonella mutagenesis assay for a variety of parameters of tumorigenicity responses. There are some subcategories of the tumorigenic response that appear to be defined more precisely by a combination of tests, as opposed to a single Salmonella result (*1*). However, there is no real net gain in information since the other three assays showed less specificity. That is, the proportion of carcinogens identified relative to the number of noncarcinogens (false positives) identified. So, in fact, there does not appear to be an advantage in the use of a combination of tests to distinguish carcinogens over the results obtained from Salmonella alone. However, I am not precluding other applications of these *in vitro* assays, and it is possible that one might want to use a combination of assays since the results from these various tests are often concordant, reinforcing the fact that these tests do identify mutagens. Therefore, the assays can play a supplementary role when used in combination, but they do not appear to play a complementary role.

We are still faced with the issue of whether or not we have really identified all of the potential mutagens among these 73 chemicals or whether there are a number of nonmutagenic chemicals that are tumorigenic. There is the implication that the Salmonella mutagenicity assay is functioning to detect a majority of carcinogenic chemicals and that either by consequence of the selection process that is involved in acquiring chemicals for testing by the NTP, or by some other quirk, we have identified a number of substances that are nonmutagenic carcinogens. I would therefore like to focus the discussion period around some questions that will relate to either mutagenicity, carcinogenicity, or risk assessment.

I would like to elicit response from the audience on the proposition that if a battery of tests do not apparently improve upon the performance of a Salmonella assay, then are we faced with the possibility of having to operationally define all mutagens on the basis of a Salmonella response. How, from a geneticist's viewpoint, is this viewed? Further, for those chemicals that have shown some dichotomous response, for example, which showed a positive response in the mouse lymphoma assay, which measures mutation at the tk locus, but were negative in Salmonella. How do we resolve dichotomies between short-term test results? Do you accept that Salmonella is detecting most mutagens? And, if so, have we reached the limits of what we can achieve *in vitro* in identifying potential mutagens?

In order to overcome some of the natural reticence that we all have to engage in public exchange, I will single one or two individuals out in the audience and ask them to respond to these questions. I'd like to start with a card-carrying geneticist and ask Dr. Larry Valcovic.

Discussion

DR. LARRY VALCOVIC, FDA: In terms of the discordance, first of all, there is not much difference between these tests. We could use any of those others as saying, Is this test detecting most mutagens? The fact that we do have a large Salmonella data base is a compelling reason for focusing on Salmonella as our baseline. I think Salmonella is probably detecting most mutagens. However, some of the other factors determining the extent to which one would desire to use supplementary tests go beyond simply detecting whether a chemical does or does not have mutagenic activity.

The term that was used in the now 10-year-old DHEW document on mutagenicity was "intrinsic mutagenic activity." This involves questions of how mutants behave and of mutant expression. This expands the question, which implied only qualitative prediction. If it's more than just the yes-no question, for example, for chromosome aberrations, we can learn something about translocations.

DR. TENNANT: Are you comfortable with accepting the repeatable negative results from a Salmonella assay that uses an S-9 as its exogenous source of metabolism?

DR. VALCOVIC: For what question?

DR. TENNANT: As a nonmutagen.

DR. VALCOVIC: As Sandy Miller was talking about in his closing remarks, this whole area of toxicity really is reducing our levels of uncertainty or modifying our levels of uncertainty. To a certain level, yes, the data from most of our experience is compelling. I think what we need to do is get more specific in terms of types of chemicals for which, in terms of carcinogenicity, the discordance lies. Clearly, if the chemical falls into that area for which there is discordance, then, yes, it's true that that compound is not mutagenic in Salmonella. But just like all areas of toxicology, we have to explore more tests to find out more about the nature of that chemical.

DR. TENNANT: Dr. Butterworth, could you share that opinion?

DR. BYRON BUTTERWORTH, CHEMICAL INDUSTRY INSTITUTE OF TOXICOLOGY: I think there are fairly simple answers for your questions. First of all, how do we define a mutagen operationally as mutagenic in the Salmonella assay? Is Salmonella detecting most mutagens? Yes, I think it is. Are we near the limits of what's achievable *in vitro*? Yes. Very simple.

I think the problem is that we need to move into the whole animal. The challenge really arises for genetic toxicology to begin to identify those things that are going on in the whole animal to help us predict carcinogenicity. Mutagenicity is one thing, predicting potential carcinogens is another. There are many different classes of nongenotoxic carcinogens and we need to try

to identify those and pick predictors that will indicate their potential carcinogenicity. And, in point of fact, very often this will only happen in the whole animal. For example, saccharin produces tumors only when you reach massive doses of 3000 mg/kg/day. Then you get hyperplasia in the bladder. To me, that hyperplasia is a good indicator of potential carcinogenicity. I think we need to begin to look at the different types of nongenotoxic carcinogens to help us as predictors. We can't mix things like TCDD with saccharin because they act in totally different ways. We can't mix the hypolipidemic agents with some of the things that cause tumors in male rat kidneys because they're acting differently. So predicting carcinogenicity isn't easy. And I think you've done a great service to help us identify that one sector, the mutagenic carcinogens. The assays are good. They work well, and now we need to get on with identifying properties of many of these other nongenotoxic carcinogens.

DR. TENNANT: I'm a little bit surprised that we are having such acceptance for the performance of Salmonella in identifying mutagens. Frankly, I expected to hear at least a cautioned of response.

DR. BUTTERWORTH: I think it's an elegant system and extremely sensitive for picking up mutational events. It's very, very good. One thing—I'm a little concerned about looking at the results of your evaluation on face value. If you're just looking at pluses and minuses, the correlation with carcinogenicity is not all that great. I'm worried that people in industry might begin to shy away from the short-term tests and say, Well, if I'm not going to pick this stuff up, why should I bother using these?

In point of fact, you found about a 50% incidence or prevalence of carcinogens in the system. When I speak with people in industry, their experience is that the number of Salmonella positives in their tests generally tend to run about 10% or less. They don't seem to have too many problems, and most people, particularly in the pharmaceutical area, use batteries of tests without many problems.

So somehow I hope that one end result of your evaluation is not to have people shy away from using the tests because it appears as though they're not predictive. I think they're very good for what they do. Let me throw this back to you. Why do you think that you have such a high prevalence of carcinogens whereas in the real world of testing that the incidence seems to be fairly low?

DR. TENNANT: Well, I think it has to do with the very origins of the rodent bioassay and the uses for the system. It has been used purposefully to attempt to guess the potential tumorigenicity of substances that for some reason have a suspected tumorigenic potential. So there's no doubt that it doesn't represent a cross-section of the chemical universe. It represents the product of a highly selected process of looking at chemicals. And I think that that bias is there and it has to be there because otherwise we would be spending a lot of money on a lot of rodents if there wasn't that selection involved. I think it has worked out very interestingly that, given the reasons for which chemicals are selected, such a high proportion have not shown carcinogenicity.

DR. AL SCHUMANN, DOW CHEMICAL: I personally feel it's important to look at multiple end points in genetic toxicology and not only Salmonella. I think as we try to get more and more sophisticated in how we assess risk we need to use each end point as a building block. As we then build a case from which we can make judgments on the preponderance of evidence on whether a material is a genotoxin or not a genotoxin. I would like to see multiple end points including an initial genetic tox screen, including getting the material into the test animal, whether it's by a micronucleus assay or some type of *in vivo* cytogenetic assay.

With respect to Salmonella, if you were only looking at that, there certainly are some instances where you may get a false picture. One case that comes to mind is methylene chloride. That material will produce mutations in Salmonella. However, it seems that as you get into the mammalian cell system and climb the phylogenetic tree, the evidence for the genetic toxicity seems to diminish, and that includes DNA-binding studies, Drosophila mutagenesis, micronucleus, UDS, those types of things. So if one would rely on only Salmonella, you might get a false picture as to the degree that a material may be genotoxic.

Going now to a practical, everyday life situation, I think this whole area is somewhat moot with our current regulatory framework. The predominating factor in the regulatory arena is not the genetic tox or lack of genetic tox. It is what is coming out of the tumor bioassay. As soon as you do get a positive out of that, irrespective of the dose level or whether you believe it's a genetic or a nongenetic mechanism, from a risk assessment framework today, that immediately goes into a unit-task assessment. We do not have the capability to adequately factor in other mechanistic considerations in the whole risk assessment process. That's really where we need to focus our attention and to evolve over the next few years as we move ahead in toxicology. We do have numerous cases where you can't say it is not a genotoxin, but yet the preponderance of evidence would suggest that the genetic activity is so weak that it may be practically nil and that other factors, such as cell toxicity, predominate. Currently we don't have a way of handling those factors. I realize that you need to be careful with those types of compounds. But we do need to work as a science towards differentiating these materials, because I think they are very different.

DR. TENNANT: Thank you. Heinrich Malling?

DR. HEINRICH MALLING, NIEHS: I know what fantastic work you and your group have done. But 73 chemicals is not very representative of the number and classes of chemicals we have in our environment. The question still remains as to how effective the Ames test is in detecting the genotoxic chemicals across the various classes of chemicals. We are presently beginning to identify chemicals which are exclusively clastogenic, such as acrylamide, and it's hard for me to see that the

Ames test would detect such chemicals. The clastogenic compounds seem not to exert any mutagenicity in the H6PRT-V79 forward mutation system. In contrast, they are mutagenic in the tk ± mouse lymphoma system where a complete or partial loss of the tk+ carrying chromosome results in a detectable mutant. So in the future it is reasonable to expect carcinogens which are also mutagens, but which cannot induce reversions in the Ames test.

DR. TENNANT: Thanks.

DR. SIDNEY GREEN, FDA: I think I would almost have to come down on the side of multitest evidence for mutagenicity as opposed to relying mainly or solely on Salmonella for very similar reasons that Heinrich just addressed. He indicated that there are substances which Salmonella clearly misses. One that comes to mind quite readily is benzene. And I think if we just look at the history in terms of numbers of compounds that have been tested in numbers of mutagenic assay systems, there are many instances in which Salmonella does not detect chemicals that are clearly carcinogens and mutagens. For that reason alone it seems to me we almost have to rely on multitest evidence again for mutagenic effects.

DR. TENNANT: Thank you.

UNKNOWN SPEAKER, HEALTH AND WELFARE, CANADA: I would also come down for multitesting for one reason. I don't see where we get off thinking that an Aroclor-induced rat liver for an S-9 system represents the real world. It represents the real world to an Aroclor-induced rat.

DR. MICHAEL RESNICK, NIEHS: How do we define a mutagen operationally? Obviously it's something that causes mutations. Except that I think most of us tend to focus on DNA damaging-type agents. We really should also be thinking about something that leads to a mutation in the classical sense, which is a permanent and heritable change in the organism. Carl Barrett alluded to this in his talk on Tuesday. Aneuploidy, of course, is a very interesting type of mutation. When we think about chromosomal aneuploidy—that is, the gain or loss of a chromosome—we're talking about a permanent change leading to the loss or gain of a considerable number of genes.

Now the target in this case can be DNA, although we lack strong evidence that DNA is a target for aneuploidy induction. In addition there are many non-DNA targets, including, for example, tubulin and proteins associated with centromeres. These are areas that we are investigating. It turns out that there are a large number of chemicals (aprotic polar solvents, e.g., methylethylketone, propionitrile, ethylacetate) that are strong inducers of aneuploidy in yeast and that act on targets other than DNA. Their effects on other systems remain to be established.

So I would say that there are several kinds of mutagens, only some of which can be detected in Salmonella. Since Salmonella lacks chromosomal organization typical of eukaryotes, it would not be able to detect agents that act on the segregational apparatus. In support of this, aprotic polar solvents are not picked up as mutagens in the Ames test.

DR. RAJENDRA CHHABRA, NIEHS: Ray, I have another provocative question after listening to several people here. How many genetic toxicity end points are necessary to predict carcinogenicity *in vivo*? Since everybody is talking about multiple toxicity end points, I think what we started with was genetic toxicity to predict *in vivo* toxicity or carcinogenicity. And here we are talking about, Okay, let's go and do some more. More genetic toxicity end points, and let's go into the *in vivo* genetic toxicity. Will the cost of doing all these tests be equal to what we do *in vivo*, or will it be more?

DR. TENNANT: Well, if you accept the fact that by some existing test or combination of tests we can identify most mutagens, we should be able to identify a substantial fraction of potential carcinogens. Then we clearly have a group of chemicals that act through some other not directly mutagenic action or through a mutagenic mode that involves some change in chromosomal number or structure. I cannot tell you how many tests it will require in order to prospectively distinguish those sorts of chemicals. Given the range of structures, toxicities, and physiologic effects represented among the nonmutagenic carcinogens, it's very unlikely that they share many common mechanisms in the way that the mutagenic chemicals may. Therefore, unless there is some uniform and fairly limited genetic substrate, like the oncogenes, for the action of nonmutagens it's very likely that it is going to take some other combination of tests to be able to recognize these sorts of chemicals prospectively.

Now, if we happen to be fortunate in that we have identified a critical substrate in the genome that is linked to the emergence of neoplastic phenotypes (if we have that in the oncogenes; and I'm not committing that we do, I'm only saying that if that turns out to be the case) there are numerous and increasingly more sophisticated approaches to answering that question. We may find some very surprising ways to address this problem in the near future. I won't preclude a technological answer to identifying chemicals that can either specifically nonmutagenically activate oncogenes, modify their expression or alter their chromosome location, and so on. I think there are obtainable answers; the costs of doing that are negotiable. It depends upon what you want to have as your product. Understanding the process might be more valuable than identifying the carcinogen.

DR. CHHABRA: If we are just focusing on fewer promising tests rather than adding more and more. . . .

DR. TENNANT: It's not just tests we're buying it seems to me, but we are buying understanding. In this whole process we have learned much more about not just about genetic toxicity of chemicals, but also about tumorigenicity.

I don't think any of us is saying that any of the *in vitro* assays can be used for risk assessment. Possibly hazard assessment, yes, but not risk assessment. We don't have the data that tells us that the response in

the *in vitro* assay can do anything beyond give us a qualitative prediction for an *in vivo* response. The data available show that if you have, for example, a positive in Salmonella assay, a negative in any future testing will not negate that positive in the Salmonella assay. Thus, a positive in the Salmonella assay has some predictive value for a positive in the rodent carcinogenicity assay, but a negative in a mammalian cell assay, whether it be gene mutation or chromosome aberrations, doesn't negate that positive.

Many believed that there are chemicals that will only produce gene mutations and will, therefore, only be detected in Salmonella assay or mouse lymphoma assay and not in a chromosome aberration assay. Also that there are only chemicals that produce chromosome aberrations and will therefore not be detected in a gene mutation assay. Therefore, to predict carcinogenicity you would need both types of assays. There's a good theoretical basis for this assumption. Unfortunately, the NCI-NTP data base doesn't support that hypothesis. We are not claiming the Salmonella assay detects all mutagens or all carcinogens. We know clearly that we are not mimicking *in vivo* metabolism and some chemicals may not be detected. Benzene might be missed, not because it's only a clastogen, but because we do not generate the active metabolite *in vitro*. There are uses for other tests, but we have to keep in mind that more tests are not necessarily better tests. We need to understand the data and understand the biology of the systems.

DR. ROY ALBERT: Well, we've run out of time and this is the question that you won't have time to discuss.

REFERENCE

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